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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> <b>C12N 15/00, 1/04, 11/00</b> <b>A01K 45/00, 61/00, C12N 5/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 90/10063</b> <b>(43) International Publication Date:</b> 7 September 1990 (07.09.90)
<b>(21) International Application Number:</b> PCT/US90/00923 <b>(22) International Filing Date:</b> 15 February 1990 (15.02.90) <b>(30) Priority data:</b> 313,594 21 February 1989 (21.02.89) US <b>(71) Applicant:</b> BIOSYS [US/US]; 1057 East Meadow Circle, Palo Alto, CA 94303 (US). <b>(72) Inventors:</b> PRUITT, Paul, L. ; 231 San Juan, Half Moon Bay, CA 94019 (US). GROVE, Donna, Irving ; 1623 Du- vall Drive, San Jose, CA 95130 (US). <b>(74) Agents:</b> GREEN, Grant, D. et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (Eu- ropean patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD AND MEDIUM FOR PACKAGING ENTOMOGENOUS NEMATODES  <b>(57) Abstract</b>  Entomogenous infective juvenile (IJ) nematodes are prepared for storage and shipment by encasing viable IJs in a thin film which is permeable to oxygen, and which contains sufficient water to maintain the IJs in a fully hydrated state. The film is rigid enough to substantially immobilize the IJs, resulting in a reduction in the amount of oxygen and food reserves required, thus extending the shelf life of IJs.		

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METHOD AND MEDIUM FOR PACKAGING  
ENTOMOGENOUS NEMATODES

Description

15 Technical Field

This application relates to the fields of packaging living organisms for storage and/or shipment, and to the use of entomogenous nematodes for insect control.

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Background of the Invention

The hazards of residual toxicity and the relative lack of specificity have rendered chemical insecticides unable to meet the requirements of modern agriculture and gardening. Lack of specificity results in destruction of beneficial insect species (e.g., honeybees) along with the destruction of target species. Residual toxicity affects organisms which encounter insecticide by ingesting dead insects, and so pass the chemical agents and their metabolites into the food chain. These problems and others have caused new interest in biological methods for pest control.

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Perhaps the oldest form of biological insect control is the use of ladybugs to combat infestation by aphids. More recently, fly populations have been reduced by release of sterilized male flies, which

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compete with fertile males and thus reduce the number of fertile eggs produced by females. Others have attempted to control insect populations through use of viruses or entomogenous fungi.

5                   Relatively recently, interest has turned to entomogenous nematodes. Nematodes make up a diverse phylum of unsegmented round worms which may be free-living or parasitic. Entomogenous nematodes parasitize insects. Typically, an entomogenous nematode in  
10 a particular developmental stage termed an "infective juvenile" or IJ enters a host insect through the alimentary canal or spiracles. Once in the host, the IJ emerges from its protective sheath and penetrates into the host's haemocoel. In the host insect's haemocoel, the nematode releases symbiotic bacteria which  
15 induce septicemia in the host, and render the host corpse suitable for nematode foraging and reproduction. The nematodes may spend several generations within the insect host, until food consumption and crowding trigger production of another IJ stage generation. The new IJs leave the host corpse in search of fresh hosts.

                  R.W. Glaser, J Exp Zool (1940) 84:1-12 reported the culturing of Neoaplectana glaseri for use  
25 in controlling Japanese beetles. N. glaseri was applied to test fields in several states, and in some cases resulted in reduction of beetle and moth grub populations (G.O. Poinar, "Nematodes for Biological Control of Insects", CRC Press, 1975).

30                   R. Gaugler, J Nematol (1981) 13:241-49 discussed the potential uses of entomogenous nematodes for control of insect populations.

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35                   However, the use of entomogenous nematodes presents several obstacles to successful commercial development. Entomogenous nematodes are highly sensitive to drying, and will eventually desiccate if held

at relative humidities of less than 100%. They are sensitive to direct sunlight, and are also somewhat prone to infection, although care must be taken that the nematodes' symbiotic bacteria is not eliminated along with any infecting agents. Accordingly, particular requirements must be met when storing or shipping entomogenous nematodes. The packaging must be able to maintain the nematodes' moisture content (e.g., by maintaining relative humidity at 100%), must provide sufficient food and oxygen to each nematode in the package (allowing for the tendency of nematodes to clump or settle), and preferably should protect the nematodes from infection by exogenous agents. IJs depend only on internal stores for food, but may metabolize their stores too quickly for long storage. Economics requires that the packaging material be inexpensive, lightweight, durable, and free from aeration restraints.

Finney, U.S. Pat. No. 4,417,545 disclosed a water-saturated foam packing material for shipping nematodes.

Bedding, U.S. Pat. No. 4,178,366 disclosed the use of entomogenous nematodes (particularly N. carpocapsae) for biological control of insects, and an anhydrous oil suspension formulation for application of nematodes to vegetation by spraying. The formulation also contained a wax component to retard water loss by nematodes in the sprayed droplets. Nematodes applied to foliage in oil suspension survived longer, as the formulation retarded desiccation.

S.R. Dutky et al, J Insect Pathol (1964) 6:417-22 disclosed the storage of a nematode designated DD-136 (possibly N. carpocapsae) in 0.1% aqueous formaldehyde at 7.1°C. The nematodes were suspended at a concentration of 50,000/mL, and 1 liter of sus-

pension was stored in an insulated gallon jug. The suspensions were oxygenated periodically.

Nelsen et al, U.S. Pat. No. 4,615,883 disclosed encapsulation of entomogenous nematodes in hydrogels, where the hydrogel capsule contains 50-98% free water in its interior, and allows diffusion of gases sufficient for respiration. The capsules may optionally be provided with a wax membrane to retard water loss. The hydrogel capsules must be sufficiently tough to resist abrasion, but pliable enough to allow release of the nematodes upon ingestion by an insect host. The capsules, having an average diameter range of 0.4-5 mm, are sprayed over an area to be treated. Hydrogels have also been employed to encapsulate microorganisms. See, e.g., Mimura et al, U.S. Pat. No. 4,450,233; Jung, U.S. Pat. No. 4,434,231; Lim, U.S. Pat. No. 4,352,883; Asai et al, U.S. Pat. No. 4,202,905; Guttag, U.S. Pat. No. 3,767,790; and Fogle et al, U.S. Pat. No. 3,541,203.

In the above formulations, the nematodes are typically formulated when in the IJ stage. This essentially eliminates the requirement for food during storage, as IJs do not feed until they unsheath, but rely on stored food. However, IJs continue to require oxygen and moisture, which must be provided by the packaging or formulation.

An alternative approach is to exploit the ability of IJs to enter a cryptobiotic state, in which metabolism is greatly reduced or halted. For example, Popiel et al, EPO 256,873 disclosed the induction of an apparent anhydrobiotic state in IJs, using carefully controlled desiccation. Upon slow desiccation, the IJs adapt and are able to survive reduced moisture levels in a state of reduced metabolic activity (anhydrobiosis). The anhydrobiotic IJs still require oxygen and moisture, but at a much lower rate than

normal "biotic" IJs. The reduction of metabolism also results in conservation of food stores. The net result is a method for storing and shipping IJs which is much less sensitive to moisture and oxygen requirements than traditional methods.

5 Yukawa et al, PCT WO 85/03412 disclosed a nematode formulation comprising a "cream" of IJs in a solution containing an antibiotic such as formaldehyde, optionally an agent to provide a high osmotic potential such as 30% sucrose, and optionally an  
10 absorbent such as activated charcoal. The formulation is stored under anaerobic conditions, and is asserted to be resistant to temperatures up to 40°C. The IJs in this formulation are presumably in an anaerobiotic state.

15 A disadvantage of some of the above formulations is that IJs may require a recovery period for their metabolism to revert to normal, before full infectivity is resumed. During this period, the nematodes may be subject to predation, and may fail to  
20 parasitize insects upon ingestion when an otherwise successful infection would normally occur. Also, the process of inducing the anhydrobiotic or cryptobiotic state can be very time consuming (e.g., 2-6 days for anhydrobiosis), and has an associated mortality rate.

#### Disclosure of the Invention

We have now invented a medium for convenient storage and shipping of entomogenous nematodes. The  
30 medium comprises a film formed from a hydrated, oxygen-permeable, reversibly cross-linked matrix containing entomogenous IJs. The film has a thickness of between about 0.5 and 5 mm, and allows oxygen to penetrate to each nematodes in sufficient quantity to  
35 assure respiration. The nematodes are restrained in an immobilized state, although cryptobiosis is not

induced. As a result of the restraint, metabolic demands for food and oxygen are reduced, which permits longer periods of storage and reduces the head space needed for oxygen supply in a sealed package.

5 Another aspect of the invention is a method for preparing the nematode film. The method comprises suspending the IJs in an aqueous solution with a sufficient amount of cross-linkable matrix material, casting the suspension into a thin sheet (about 0.5-5 mm thick), and cross-linking the matrix to form a  
10 sheet.

Another aspect of the invention is the method of controlling an insect population, by reversing the cross-linking of a nematode film, freeing the  
15 nematodes, and applying the freed nematodes to an area having insects to be controlled. Another aspect of the invention is the method wherein the nematode film is applied to an area having insects to be controlled, and is then uncross-linked to free the nematodes. In  
20 the practice of the latter method, the film may advantageously include photoprotective agents to protect nematodes from direct sunlight, and may be designed to maintain a high level of moisture in the application area for an extended period of time.

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#### Modes of Carrying Out The Invention

##### A. Definitions

The term "entomogenous nematode" refers to  
30 nematodes which parasitize and kill insects. Presently preferred entomogenous nematodes are derived from the Family Steinernematid and Heterorhabditid nematodes, particularly Neoplectana carpocapsae, N. bibionis, N. glaseri, and H. heliothidis.

35 The term "infective juvenile" or "IJ" refers to an entomogenous nematode in the infective third

larval stag . IJs are characterized by retention of  
th s cond stage cuticle or sheath after molting to  
third stag . IJs do not eat, but depend on internal  
food stores. They are capable of substantial vertical  
and horizontal migration, and are generally the only  
nematode stage capable of establishing a productive  
infection in insects.

The term "cryptobiosis" refers to a state of  
dormancy in which metabolism essentially ceases. In  
this state, the IJ fails to respond to physical  
manipulation, and appears inert upon inspection.  
Cryptobiotic IJs may be stored for long periods with-  
out air or food, but generally require a recovery  
period prior to reestablishment of full infectivity.  
"Anhydrobiosis" refers to a cryptobiotic or semi-  
cryptobiotic state which is induced by gradual desic-  
cation of IJs. In the anhydrobiotic state, IJs gener-  
ally coil and cease movement, and may survive removal  
of most of their body water content. Anhydrobiotic  
IJs may still require oxygen, but at a rate greatly  
reduced from motile IJs.

The term "reversibly cross-linkable matrix  
material" refers to a substance which may be cross-  
linked to form a relatively rigid gel. The matrix  
material must be capable of permitting diffusion of  
gases sufficient for nematode respiration while  
immobilized, must retain sufficient water to prevent  
desiccation, and must be substantially non-toxic to  
the nematodes employed in the film. The oxygen perme-  
ability will of course vary with the species of nema-  
tode selected, the degree of immobilization, the con-  
centration of IJs in the film, and the thickness of  
the film cast. However, by immobilizing the IJs  
within the film, we have found that oxygen diffusion  
rates may be used which are far lower than the oxygen  
diffusion rates required for non-immobilized, biotic

nematodes. The degree of hydration should be about 50-90% in order to assure non-desiccation. The matrix material must be capable of being cross-linked to a degree sufficient to substantially immobilize the IJs. The degree of cross-linking may be estimated by the rigidity of the resulting film, e.g., using a durometer. The films of the invention are rigid enough that <2% of the entrained nematodes are able to migrate out of the gel within 72 hours. The cross-linked film must be capable of being unlinked or dissolved in a non-toxic solvent to release the nematodes. Finally, the cross-linking and unlinking conditions must be mild enough for the IJs to tolerate. Reversibly cross-linkable matrix materials useful in the present invention include sodium alginate, carrageenan, gelatins, xanthan gums, and the like. The presently preferred matrix material is alginic acid. Alginic acid suspensions are cross-linked by the addition of  $\text{Ca}^{++}$ , and are unlinked by removal of  $\text{Ca}^{++}$ , e.g., by addition of citrate and/or EDTA.

The phrase "limits water loss" refers to the reduction in water loss by evaporation. In general, the water loss should be low enough that the film is not desiccated in the container during storage or shipment. For a  $1 \text{ ft}^2$  film, a water loss of about 0.15 g/day at ambient temperatures is acceptable.

#### B. General Method

Infective juvenile nematodes are prepared by any acceptable means, such as the methods disclosed by Glaser, supra, or Bedding, U.S. Pat. No. 4,334,498.

A suspension of IJs in buffered aqueous solution is prepared with a suitable concentration of matrix material. The concentration of IJs may range from about  $1 \times 10^5/\text{mL}$  to about  $6 \times 10^5/\text{mL}$ , preferably about  $3 \times 10^5/\text{mL}$ . The concentration of matrix

material depends upon the particular material selected, and may be determined by routine experimentation. Where alginic acid is employed, the preferred concentration is about 0.75% to about 10%, preferably about 2% when cast. In general, the suspension is prepared to provide sufficient viscosity that it may be cast on a screen of opening size <1-2 mm or on a solid support. The suspension is mixed well and cast as a film of thickness 4 to about 6 mm, and cross-linking is initiated. Where a cross-linking agent is required, it may be added after casting, or immediately prior to casting. In the case of alginic acid, the preferred cross-linking agent is a divalent metal cation, preferably  $\text{Ca}^{++}$ . About 0.5 M to 2.0 M  $\text{CaCl}_2$  is added to the cast film, and the gel allowed to harden to provide a nematode film of the invention. The film may then be removed from the support and cut to an appropriate size for packaging. The film may be prepared embedded in the screen, and the entire screen and film composition may be cut to appropriate size.

The film may be stored in a polymer bag or bottle which is capable of transmitting oxygen while limiting water loss by evaporation. Water loss should be limited to  $\leq 0.15$  g/day per square foot of matrix. Oxygen permeability should provide about 70 cc of  $\text{O}_2$  day per square foot of matrix.

Unlinking is accomplished in a manner dependent upon the particular matrix material selected. In the case of alginic acid, the divalent metal cation is removed, typically by complexation with EDTA and/or citric acid. The film may be immersed in a suitable solution, and stirred until the film has substantially disintegrated. The resulting suspension contains viable, biotic IJs, and non-toxic matrix materials, and may be applied directly to an area having insects to be controlled.

Viability of nematodes may be assessed microscopically, by observing the reaction to prodding with a dissection needle. Infectivity is traditionally assessed by applying the IJs to Galleria mellonella larvae and noting the rate of mortality. Normally, at least 40% of the G. mellonella larvae will be dead within 48 hours of application.

### C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

#### Example 1

(Preparation of Nematode Film)

(A) Neoaplectana carpocapsae IJs were cleaned and disinfected by washing with a suitable disinfectant, such as dilute hypochlorite.

Deionized water (7.5 Kg), Proxel™ (a biocide, 8 g), Keltone™ HV® (an alginic acid analog, 300 g), Min-U-Gel® (a montmorillonite clay extender, 225 g), and Waterlock™ G400® (a modified starch humectant, 15 g) were blended in a mixing vessel with strong agitation to form a uniform slurry of high viscosity. To this slurry was added an aqueous suspension of disinfected IJs ( $6.3 \times 10^5$  IJ/mL, 8048 g), and the mixture blended slowly under very mild agitation.

The slurry (80 g) was then applied to one square foot of fiberglass window screening having about 15 strands/inch, and was spread to a uniform layer 3-6 mm thick. The entire sheet was then immersed in an aqueous solution of  $\text{CaCl}_2$  (1.6 M: calcium phosphate is also acceptable), and held until the gel had set (about 30-60 seconds). The sheet was then

transferred to a pure water bath for about 10-30 seconds to remove excess calcium.

The sheet is then allowed to drip dry, and is packaged in bags of semipermeable polymer film, or in bottles having loose caps or semipermeable film lids. The package need only supply about 70 cc of oxygen per day to support the IJs immobilized in one square foot of film. In contrast, an equal number of free-swimming IJs in suspension would require about 180 cc of oxygen per day.

The product contains fully biotic entomogenous nematode IJs, may be stored under refrigeration if desired. If stored at ambient temperature, the product exhibits adequate viability for over 30 days. If stored under refrigeration (e.g., at 5°C), the product will be useful even after six months of storage.

(B) A nematode film was prepared as in part A above, but substituting H. heliothidis for N. carpocapsae, and using calcium phosphate instead of calcium chloride.

#### Example 2

To use the nematode film, the entire film was immersed in a 40 oz aqueous solution containing sodium citrate 10% and EDTA 1%. After about 15 minutes, the film disintegrated, and was ready for dilution.

The suspension is diluted for further use. For use with a "back-pack" type sprayer, the suspension is diluted with about 1.5 gallons of water. Use with a hose-end type sprayer dilutes the suspension with about 200 gallons. Alternatively, the suspension may be added to 3 gallons of water in a watering can for use with potted plants. The suspension is sufficient to cover about 550 square feet.

If desired, the film container may be dimensioned to provide sufficient volume for the solution for unlinking the film, so that the film may be dissolved without removing it from the container. Water is simply added to the container up to a fill line, a packet containing sodium citrate and EDTA is added, and the container is closed and shaken vigorously.

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WHAT IS CLAIMED:

1. A nematode film for storage and shipment of entomogenous infective juvenile nematodes, which film comprises:

an oxygen-permeable, reversibly cross-linked matrix having a thickness of about 0.5 to about 5 mm, having a water content sufficient to preserve infective juvenile nematodes in a non-desiccated state, and having sufficient rigidity to substantially immobilize infective juvenile nematodes; and viable entomogenous infective juvenile nematodes immobilized in said cross-linked matrix in a non-cryptobiotic state.

2. The film of claim 1, wherein said reversibly cross-linked matrix comprises calcium alginate.

3. The film of claim 2 wherein said entomogenous nematodes are selected from Steinernematid and Heterorhabditid infective juveniles.

4. The film of claim 3 wherein the infective juveniles are of the species N. carpocapsae.

5. The film of claim 3 wherein the infective juveniles are of the species H. heliothidis.

6. The film of claim 3 wherein the infective juveniles are of the species N. bibionis.

7. The film of claim 3 wherein the infective juveniles are of the species N. glaseri.

8. A method for preparing a nematode storage film, which method comprises:

suspending entomogenous infective juvenile nematodes and reversibly cross-linkable matrix material in aqueous suspension;

forming a film from said aqueous suspension, said film having a thickness of about 0.5 to about 5 mm, wherein said nematodes are present within said film; and

cross-linking said matrix material to provide a film which mechanically immobilizes said nematodes.

9. The method of claim 8, wherein said reversibly cross-linkable matrix material comprises alginic acid.

10. The method of claim 9, wherein said cross-linking comprises adding  $\text{Ca}^{++}$ .

11. A package of entomogenous infective juvenile nematodes, which package comprises:

an oxygen-permeable, reversibly cross-linked matrix having a thickness of about 0.5 to about 5 mm, having a water content sufficient to preserve infective juvenile nematodes in a non-desiccated state, and having sufficient rigidity to substantially immobilize infective juvenile nematodes; and viable entomogenous infective juvenile nematodes immobilized in said cross-linked matrix in a non-cryptobiotic state;

a container dimensioned to receive said matrix, wherein said container permits entry of about 70 cc  $\text{O}_2$  per square foot of matrix, and limits water loss by evaporation.

12. The package of claim 11, wherein said matrix has a surface area of about 0.5 square feet.

5 13. The package of claim 11 wherein said matrix comprises alginate.

14. The package of claim 13 which further comprises an amount of sodium citrate and EDTA sufficient to dissolve said matrix.

10 15. The package of claim 14 wherein said container has a volume of at least 20 oz.

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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00923

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) <sup>6</sup>  
 According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC (5): C12N 15/00, C12N 1/04, C12N 11/00, A01K 45/00, A01K 61/00, C12N 5/00  
 US: 800/2, 435/260, 240.22, 174, 119/2,3

**II. FIELDS SEARCHED**

Minimum Documentation Searched <sup>7</sup>	
Classification System	Classification Symbols
US	800/2 435/260, 240.22, 174 119/2,3

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>

USPTO AUTOMATED PATENT SYSTEM-FILE USPAT, JAMBO JPOABS DIALOG FILES-BIOSIS PREVIEW CLAIMS  
 TM/U.S. PATENTS, INPADOC/FAMILY AND LEGAL STATUS, WORD PATENTS INDEX, CHINESE PATENT ABS IN  
 ENGLISH BIOTECHNOLOGY ABSTRACTS, SEE ATTACHMENT FOR SEARCH TERMS.

**III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>**

Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X/Y	US, A, 4,615,883 (NESLEN) 07 October 1986	1-11,13,14/1-15
Y	US, A, 4,765,275 (YUKAWA) 23 August 1988	1-15
Y,P	US, A, 4,814,274 (SHIOYA) 21 March 1989	1-15
Y,P	US, A, 4,806,355 (GOOSEN) 21 February 1989	1-15
Y	US, A, 4,647,536 (MOSBACH) 03 March 1987	1-15
Y	US, A, 4,803,168 (JARVIS) 07 February 1989	1-15
Y	US, A, 4,798,786 (TICE) 17 January 1989	1-15
Y	US, A, 4,778,749 (VASINGTON) 18 October 1988	1-15
Y	US, A, 4,663,286 (TSANG) 05 May 1987	1-15
Y	US, A, 4,407,957 (LIM) 04 October 1983	1-15
Y	US, A, 4,417,545 (FINNEY) 29 November 1983	1-15
Y	US, A, 4,352,883 (LIM) 05 October 1982	1-15
Y	US, A, 4,391,909 (LIM) 05 July 1983	1-15
Y	US, A, 4,409,331 (LIM) 11 October 1983	1-15

<sup>\*</sup> Special categories of cited documents: <sup>10</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

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**IV. CERTIFICATION**

Date of the Actual Completion of the International Search

12 APRIL 1990

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

21 MAY 1990

Signature of Authorized Officer

CHRISTOPHER LOW

*Ngan Ho Nguyen*  
 Ho NGUYEN

ATTACHMENT TO FORM PCT/ISA/210, PART II  
CLASSIFICATION OF SUBJECT MATTER:

SEARCH TERMS:

nematode  
nematodes  
package

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